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Antimalarial activity of *Ageratum conyzoides* in combination with chloroquine and artesunate

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ABSTRACT

Objective: To determine the suppressive and curative activity of aqueous leaf extract of *Ageratum conyzoides (A. conyzoides)* in combination with chloroquine and artesunate, respectively against *Plasmodium berghei* infection in mice. **Methods:** Using malaria (*Plasmodium berghei*) infected albino mice of both sexes, aqueous extracts of *A. conyzoides* in combination with chloroquine and artesunate were tested for antimalarial activity, respectively. Four-day suppressive test and Rane's curative test were carried out. **Results:** Suppressive tests showed significant dose dependent reduction in parasitemia level produced by the extract-chloroquine and extract-artesunate combinations. Suppressive activities of both extract-drug combinations were greater than the individual drugs alone. Extract-chloroquine (100:5) produced the highest suppressive effect (98% suppression). Curative tests showed absolute survival in two extract-drug combinations. Two extract-drug combinations produced higher curative effects than the individual drugs alone. The highest dose combinations of extract-chloroquine (100:5) and extract-artesunate (100:5) produced absolute parasitemia clearance (cure) in the infected mice. **Conclusions:** The study indicated that aqueous extract of *A. conyzoides* had the ability to potentiate the antimalarial activity of chloroquine and artesunate against induced plasmodiasis in mice. It contributes a lot in the malaria endemic and poverty stricken tropics.

1. Introduction

Malaria continues to pose a serious threat to human population of the tropical and subtropical regions. The appearance of drug resistant strains of malaria parasite has materially worsened this danger, and this has become a major stumbling block to economic development and tourism in the affected areas[1]. Despite the increasing threat of malaria to lives especially in Africa, success in controlling the disease is still possible[2]. Different approaches have been explored to control malaria including vector control methods, chemotherapy, exploring evidence of immunity, *etc*[3]. Chemotherapy is the main stay of malarial control. The previously efficacious chloroquine has substantially failed as a prophylactic and therapeutic antimalarial in many endemic countries of Africa, Asia and South America due to a corresponding spread of chloroquine resistant *Plasmodium berghei (P. berghei)* strains with mutant alleles for chloroquine resistance transporter protein (pfcrt T76) and multidrug resistant glycoprotein (pfmdr IY86)[4,5]. Consequently the diminished potency of chloroquine above the 25% failure in many countries including Nigeria has paved way for the use of other antimalarial drugs. Unfortunately, these alternative antimalarial drugs including sulfadoxine-pyrimethamine combination, mefloquine and halofantrine are also resisted by *P. falciparium*[6,7]. Combination drug regimens have become the practice of choice because of their increased therapeutic efficacy over monotherapy and the other benefits including decreased cytotoxicity, delay or prevention of the development of drug resistance[8]. It is widely reported that artemisinins in combination with other antimalarials have distinct mode of action and remain the only anti-malarial regimens, though resistance to artemisinin monotherapy is emerging[9,10]. However, most of these regimens are administered in low doses and it

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is difficult in implementation especially in rural areas of developing countries due to affordability and accessibility constrains[11]

This scenario has re-awakened interest in the exploration of medicinal plants from which antimalarials such as quinine and artemisinin were found[12]. Plants, undoubtedly, play an important role in African indigenous system of medicine, especially in the rural areas with limited access to modern medical and health facilities[13]. Herbal drugs or their extracts are prescribed widely, even when their biological active compounds are unknown[14]. World Health Organization (WHO) estimates that up to 80% of the African population use traditional medicine especially plant remedies in the management of diseases including malaria[15]. In Nigeria, mono or polyherbal mixtures have been employed in treatment of malaria symptoms[16].

Ageratum conyzoides (A. conyzoides) (Family: Asteraceae) is one of such plants with folkloric reputation as antimalarial agent. It is an annual herbaceous plant widely distributed in the tropics as a herbal weed. Extracts of the plant have been used in traditional medicines as remedies against wounds and burn, microbial infections, arthrosis, headache, inflammation dyspnea, pain, asthma, spasms, gynaecological diseases, leprosy and other skin diseases[17]. In Africa, *A. conyzoides* is also traditionally used to treat fever, rheumatism, headache, malaria, pneumonia, wounds, burns and colic[18]. An earlier pharmacology study of the plant showed significant positive antimalarial activity in rats[19].

This study was aimed at evaluating the antimalarial activity of the aqueous leaf extract of *A. conyzoides* when in combination with standard antimalarials: chloroquine and artesunate, respectively.

2. Materials and methods

2.1. Chemicals and test agents

Methanol, n-hexane, Chloroform, Tween 80 (all Sigma Aldrich, Germany), Chloroquine (Neimeth®, Nigeria), Artesunate (Emzor®, Nigeria), distilled water. All reagents were of analytical grade.

2.2. Plant material

Fresh leaves of *A. conyzoides* were collected from wild plants growing around farmlands in University of Nigeria, Nsukka in September 2008. The plant was identified by Mr. Paulinus Ugwuozor, a taxonomist in the Department of Botany, University of Nigeria, Nsukka. A voucher specimen was preserved at the herbarium of Pharmacognosy Department, University of Nigeria, Nsukka.

2.3. Preparation of extract

The leaves of the plant were cleaned, shade dried for four days and pulverized to coarse powder. One kilogram (1 kg) of the dried leaf powder was extracted with 4 liters of distilled water by cold maceration method. The extract was preserved in a refrigerator and later lyophilized at the National Institute of Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. The resultant dried extract was designated as aqueous extract of *A. conyzoides* and kept at cool storage before use.

2.4. Animals

Adult male and female Swiss albino mice weighing between 18-25 g obtained from the Animal Facility Centre of NIPRD were used. The animals were observed under light/dark cycle in metabolic cages in a well ventilated [average room temperature of (28±4)℃] rodent cubicle. They were fed with mice pellet diet and given free access to clean drinking water. The animals were kept in well disinfected rooms (to avoid any form of parasite infections) and allowed to acclimatize for two weeks before the start of the experiment. All the animals were conducted according to regulations of the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.5. Rodent parasite

Chloroquine sensitive *P. berghei* (NK65 Strain) was sourced from the National Institute for Medical Research (NIMR), Lagos and maintained in NIPRD by serial passaging in mice.

2.6. Antiplasmodial activity

2.6.1. Suppressive effect of A. conyzoides separately combined with chloroquine and artesunate

The suppressive effect of aqueous extract of *A. conyzoides* in combination with chloroquine and artesunate, respectivelyagainst *P. berghei* infection in mice was determined using Peters' 4-day suppression test procedure[20]. A donor mouse densely infected with parasites was anaesthetized with chloroform and the blood was collected through cardiac puncture. The presence of parasitemia was established by microscopic examination of a thin blood film. One mL blood was diluted with normal saline to 20 mL. A total of 0.2 mL diluted blood was injected intraperitoneally into 54 healthy mice. The infected animals were randomly divided into 9 groups namely three *A. conyzoides*-chloroquine groups, three *A. conyzoides*-artesunate groups, chloroquine group, artesunate group and negativecontrol group. The animals were treated shortly after inoculation on day zero (D_o). *A. conyzoides*-chloroquine groups were given chloroquine at 5 mg/kg concurrently with *A. conyzoides* at 25, 50 and 100 mg/kg, p.o., respectively. *A. conyzoides*-artesunate groups were given artesunate at 2 mg/kg and *A. conyzoides* at different dosage as above. Chloroquine group received chloroquine at 5 mg/kg. Artesunate group received artesunate at 2 mg/kg. And negative control group received 0.2 mL of distilled water. Oral route

was used for all administration. Treatment continued daily (D_1-D_3) until D_4 when thin film from the tail blood of each mouse was made on a microscopic slide. The slides were stained with leishman stain, and examined under the microscope. Parasitemia level was determined by counting average number of parasites in 10 fields of at least 1 000 erythrocytes. Percentage parasitemia inhibition was then calculated.

2.6.2. Combined curative effect of A. conyzoides separately combined with chloroquine and artesunate

Rane's curative test procedure was used[21]. The inoculation and treatment protocols used for Rane test were similar to suppressive test procedure, except that treatment started on the D_3, that is, 72 hours after infection was established. The number of animals and the groups were the same as above. On D_3, pretreatment blood smear of each mouse was made and examined under the microscope. *A. conyzoides*-chloroquine groups received 10 mg/kg, p.o. of chloroquine, and *A. conyzoides* at 25, 50, and 100 mg/kg, p.o., respectively. *A. conyzoides*-artesunate groups received 4 mg/kg of artesunate, and *A. conyzoides* at different dosage as above. Chloroquine group received chloroquine at 10 mg/kg. Artesunate group received artesunate at 4 mg/kg. And negative control group received 0.2 mL of distilled water. Treatment continued daily (D_4-D_6) until D_7 when post treatment blood smears of each mouse was made on a microscopic slide and examined microscopically. The blood smear was also collected on the D_{14} and examined microscopically. The animals were observed for 30 days and the mean survival time of each group was recorded[22].

2.7. Statistical analysis

Results were expressed as mean±standard errors of mean (SEM). Analysis of variance (ANOVA) was used to analyze differences among groups. Subgroup analysis (post hoc) was conducted using LSD. Then a 2 tailed significant of 0.05 level was used as significant difference.

3. Results

3.1. Suppressive tests

Table 1 showed results of suppressive tests by artesunate, chloroquine and Ageratum extract-drug combinations. Percentage suppression showed significant (P<0.05) suppression for all treatments compared to control. Suppressive effects produced by the extract-drug combinations were higher than chloroquine and artesunate alone. Also the suppressive effects of extract-chloroquine combination were higher than those produced by extract-artesunate combination at equivalent dose combinations. All suppressive effects were dose-dependent in the extract-drug combinations.

3.2. Curative tests

Curative effects test showed absolute survival times for all test treatments including extract-drug combinations. Duration of survival was similar to all treatments as all animals survived after 30 days. All animals in the control died before the 12th day. Cure from plasmodium parasitemia was significant (P<0.05) in all treatments when compared with control. *A. conyzoides* -chloroquine (100:10) and *A. conyzoides* -artesunate (100:4) produced absolute parasitemia clearance (0 cell/mL of *P. berghei*) before the 14th day. Artesunate produced a better clearance than chloroquine on day 7 and 14 of treatment.

4. Discussion

This study had demonstrated the inherent ability of the aqueous leaf extract of *A. conyzoides* to potentiate the suppressive and cure rate of chloroquine as well as artesunate *in vivo* in mice infected with *P. berghei*. An earlier study had shown the positive plasmodial suppressive activity of *A. conyzoides*[19] and agents with suppressive activity against *P. berghei* for antimalarial activity[23].

Co-administration of *A. conyzoides* with prophylactic dose of chloroquine (5 mg/kg), and maintenance dose of artesunate (2 mg/kg), resulted in dose dependent chemosuppression of parasitemia, that was greater than the effects of chloroquine, artesunate or selected doses of the plant extract used singly in *P. berghei* infection in mice[19]. This additive effect was seen in all the combination doses used. The increase in parasitemia clearance with increase in dose of *A. conyzoides* in each combination showed there were probably no antagonistic effects produced by the herb-drug combination.

In the curative test, co-administration of selected doses of the aqueous extract with therapeutic dose of chloroquine, though did not totally clear the parasite on the day 7 post treatment, reduced the parasitemia count to much lower levels compared to chloroquine alone. Extract-artesunate combinations also produced excellent parasitemia reduction, three days post-treatment (Day 7) compared to reduction of artesunate drug alone. These may suggested potent synergistic anti-malarial activity between the extract and these standard drugs, chloroquine and artesunate.

Till day 14, no form of recrudescence was seen in all the drug combinations, as parasitemia clearance was consistent till the 14th day. This showed potent and long term antimalarial effect of the herb-drug combinations. With curative dose of artesunate (4 mg/kg), the combination totally cleared the parasitemia at extract dose of 100 mg/kg on day 7, compared to artesunate or chloroquine alone. The combination (extract-artesunate, 100:4) maintained the absolute cure rate on day 14 and prolonged recrudescence time unlike artesunate alone which had already shown recrudescence on day 14. This is in line with reports of Iwalokun *et al*[24]. This is one of the reasons for initiating artemisinin combination therapy (ACT) in acute

Table 1

Suppressive effect of *A. conyzoides* combined with chloroquine and artesunate against *P. berghei* infection in mice.

Treatment	Parasitemia count on D_4	Suppression (%)
Control (Distilled water)	25.00±0.86	–
25 mg/kg *A. conyzoides* + Chloroquine	3.20±0.27*	87.20
50 mg/kg *A. conyzoides* + Chloroquine	2.00±0.23*	91.60
100 mg/kg *A. conyzoides* + Chloroquine	0.30±0.04*	98.80
25 mg/kg *A. conyzoides* + Artesunate	3.20±0.23*	87.20
50 mg/kg *A. conyzoides* + Artesunate	3.00±0.24*	88.00
100 mg/kg *A. conyzoides* + Artesunate	1.80±0.19*	92.80
Chloroquine	3.80±0.34*	84.80
Artesunate	4.50±0.90*	82.00

* $P<0.05$ in Dunnett-T tests; D_4 represents 4 days after inoculation respectively; n=number of rats per treatment group.

Table 2

Curative effect of *A. conyzoides* with chloroquine and artesunate against *P. berghei* in mice.

Treatment	Parasitemia count			Survival time (days)
	D_3	D_7	D_{14}	
Control (Distilled water)	24.00±0.77	36.00±1.15	47.00±2.62	11.7
25 mg/kg *A. conyzoides* + Chloroquine	22.00±0.97	6.00±0.52*	3.00±0.24*	30.0
50 mg/kg *A. conyzoides* + Chloroquine	27.00±1.13	1.90±0.68*	0.20±0.03*	30.0
100 mg/kg *A. conyzoides* + Chloroquine	24.00±0.68	0.20±0.37*	0.00±0.00*	30.0
25 mg/kg *A. conyzoides* + Artesunate	26.30±0.82	4.40±0.36*	2.50±0.20*	30.0
50 mg/kg *A. conyzoides* + Artesunate	23.10±0.65	3.00±0.34*	1.97±0.11*	30.0
100 mg/kg *A. conyzoides* + Artesunate	24.00±0.82	0.00±0.00*	0.00±0.00*	30.0
Chloroquine	23.20±1.24	6.70±0.38*	5.00±0.39*	30.0
Artesunate	22.00±1.18	5.00±0.37*	12.00±0.58*	30.0

* $P<0.05$ compared to control using Dunnett-T tests on D_7 and D_{14}; D_3, D_7, D_{14} represents 3, 7, 14 days after inoculation respectively.

uncomplicated malaria. Thus a combination of extract of *A. conyzoides* and artesunate would produce excellent cure rates and reduce recrudescence. The mean survival time of the treated groups in both cases was more than 4 weeks unlike that of the untreated control which died before two weeks. It further confirmed the fact that the herb-drug combinations were effective in parasitemia reduction to non-fatal levels even after long periods of post-treatment.

The findings of this study are very similar with other reports on other antimalarial herb-drug interactions that showed potentiating effect of herbs on the antimalarial action of some standard drugs[25-29]. The potentiating antimalarial effect of the extract of *A. conyzoides* on the antimalarial activity of chloroquine and artesunate, might probably be due to prolongation of elimination half life of these drugs as reported[25], or synergistic action with chloroquine in inhibiting hematin formation as reported[30,31]. These assumptions however form the basis of our future research on this plant.

This study demonstrated the ability of aqueous leaf extract of *A. conyzoides* to potentiate the antimalarial activities of chloroquine and artesunate when used as an adjuvant. Further development of *Ageratum* for this purpose would no doubt provide cost effective strategy of malaria control especially in developing countries where poor affordability and accessibility of orthodox drugs are primary barrier to healthcare. More studies are needed to elucidate the exact mechanism of action of *A. conyzoides* coupled with toxicity testing for the assessment of its drug-extract efficacy and safety.

Conflict of interest statement

We declare that we have no conflict of interest.

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